Gabexate Mesilate, a Synthetic Protease Inhibitor, Inhibits Lipopolysaccharide-Induced Tumor Necrosis Factor-α Production by Inhibiting Activation of Both Nuclear Factor-κB and Activator Protein-1 in Human Monocytes

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ABSTRACT
Gabexate mesilate, a synthetic protease inhibitor, was shown to be effective in treating patients with sepsis-associated disseminated intravascular coagulation (DIC) associated with sepsis (Taenaka et al., 1983). We previously demonstrated that gabexate mesilate reduced tumor necrosis factor-α (TNF-α) production in rats administered endotoxin by inhibiting TNF-α production in rats. In the present study, we analyzed the mechanism(s) by which gabexate mesilate inhibited TNF-α production in human monocytes in vitro. Gabexate mesilate inhibited the production of TNF-α in monocytes stimulated with LPS. Gabexate mesilate inhibited both the binding of nuclear factor-κB (NF-κB) to target sites and the degradation of inhibitory κBα. Gabexate mesilate also inhibited both the binding of activator protein-1 (AP-1) to target sites and the activation of mitogen-activated protein kinase pathways. These observations strongly suggest that gabexate mesilate inhibited LPS-induced TNF-α production in human monocytes by inhibiting activation of both NF-κB and AP-1. Inhibition of TNF-α production by gabexate mesilate might explain at least partly its therapeutic effects in animals given LPS and those in patients with sepsis.

On stimulation with lipopolysaccharide (LPS), monocytes release a variety of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (Morrison and Ryan, 1987). TNF-α plays a critical role in the development of disseminated intravascular coagulation (DIC) associated with sepsis (Levi et al., 1999). TNF-α also contributes to activated neutrophil-induced endothelial injury, not only by activating neutrophils (Klebanoff et al., 1986) but also by activating endothelial cells leading to an increase in the expression of endothelial leukocyte adhesion molecules such as E-selectin and intercellular adhesion molecule-1, both of which enable activated neutrophils to adhere to the endothelial cell surface (Mulligan et al., 1991).

Gabexate mesilate is a synthetic protease inhibitor that has anticoagulant activities (Tamura et al., 1977). Gabexate mesilate was shown to be effective in treating patients with DIC associated with sepsis (Taenaka et al., 1983). We previously demonstrated that gabexate mesilate reduced pulmonary vascular injury as well as coagulation abnormalities in rats administered endotoxin by inhibiting TNF-α production (Murakami et al., 1996). TNF-α plays a critical role in the development of acute respiratory distress syndrome (ARDS) by activating neutrophils in patients with sepsis (Shanley et al., 1995). Because ARDS is associated with a high mortality in patients with sepsis (St. John and Dorinsky, 1993), inhibition of TNF-α by gabexate mesilate could be useful in reducing the mortality of such patients by inhibiting both pulmonary vascular injury and coagulation abnormalities. However, the precise mechanism(s) by which gabexate mesilate inhibits TNF-α production by monocytes is not well understood at present.

Nuclear factor-κB (NF-κB), a transcription factor, is critically involved in the regulation of monocyctic production of proinflammatory cytokines, such as TNF-α and interleukin-1β (Baldwin, 1996). The most abundant form of NF-κB is a heterodimer composed of p50 and p65 subunits (Baldwin, 1996). In unstimulated monocytes, NF-κB is localized in the cytoplasm as an inactive complex with inhibitory κBα. Upon stimulation, NF-κB is released from the inhibitory molecule and translocates to the nucleus, where it binds to specific DNA sequences and activates gene expression.

NF-κB plays a critical role in the development of sepsis-associated DIC (Shanley et al., 1995). NF-κB activation is mediated by activator protein-1 (AP-1), a transcription factor that is composed of Jun and Fos subunits. AP-1 activation is regulated by mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinases (p38 MAPK).

In the present study, we examined the mechanisms by which gabexate mesilate inhibited TNF-α production in human monocytes. We demonstrated that gabexate mesilate reduced LPS-induced TNF-α production in vitro. Gabexate mesilate inhibited the binding of NF-κB to target sites and the degradation of inhibitory κBα, as well as the binding of activator protein-1 to target sites and the activation of mitogen-activated protein kinase pathways. These observations strongly suggest that gabexate mesilate inhibits TNF-α production in human monocytes by inhibiting activation of both NF-κB and AP-1. Inhibition of TNF-α production by gabexate mesilate might explain at least partly its therapeutic effects in animals given LPS and those in patients with sepsis.

ABBRiEVATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; DIC, disseminated intravascular coagulation; ARDS, acute respiratory distress syndrome; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; AP-1, activator protein-1; IκBα, inhibitory κBα; PBMC, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; TLR, toll-like receptor.
cytosol as an inactive form bound to inhibitory \( \kappa \)B (\( \kappa \)B) (Finco and Baldwin, 1995). On stimulation with LPS, \( \kappa \)B undergoes phosphorylation, ubiquitination, and proteolytic degradation, permitting NF-\( \kappa \)B to translocate to the nucleus to initiate gene transcription (Baldwin, 1996).

Members of the mitogen-activated protein kinase (MAPK) family are also important in the signal transduction system to initiate gene transcription (Baldwin, 1996). Antigen-presenting \( \kappa \) in human monocytes (Aosasa et al., 2001). However, little is known about the detailed molecular mechanisms by which gabexate mesilate inhibits the activation of NF-\( \kappa \)B. Furthermore, it is not clear whether gabexate mesilate inhibits the activation of AP-1 in human monocytes stimulated with LPS.

In the present study, we examined whether gabexate mesilate inhibits LPS-induced TNF-\( \alpha \) production in human monocytes by inhibiting the activation of NF-\( \kappa \)B and AP-1.

Materials and Methods

Materials. Gabexate mesilate was a generous gift from the Ono Pharmaceutical Company (Osaka, Japan). LPS (Escherichia coli, serotype 055:B5) was purchased from Difco (Detroit, MI). RPMI 1640 medium was obtained from Invitrogen (Carlsbad, CA). Supplemented calf serum was from Hyclone Laboratories (Logan, UT). Antibodies against \( \kappa \)B and phosphorylated \( \kappa \)B (Ser32), phosphorylated JNK (Thr183/Tyr185), and phosphorylated p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology Inc. (Beverly, MA). DIG gel shift kit was from Roche Diagnostics (Mannheim, Germany). Double-stranded oligonucleotides with consensus sequences of NF-\( \kappa \)B and AP-1 were obtained from Promega (Madison, WI). All other reagents used were of analytical grade.

Monocyte Preparation and Incubation. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteer blood donors as described previously (Uchiba et al., 1997). The cell preparations were >90% monocytes, as determined by trypan blue dye exclusion test. Human monocytes were incubated in RPMI 1640 medium supplemented with 1% supplemented calf serum. The cells were stimulated with LPS in the presence or absence of gabexate mesilate.

Measurement of TNF-\( \alpha \). Human monocytes (5 \( \times \) 10\(^6\) cells/assay) were stimulated with LPS (100 ng/ml) or (1 ng/ml) for 4 h in the presence or absence of various concentrations of gabexate mesilate. Concentrations of TNF-\( \alpha \) in supernatant fractions were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit for human TNF-\( \alpha \) (Biosource International, Camarillo, CA).

Western Blot Analysis. Human monocytes (2 \( \times \) 10\(^6\) cells/assay) were stimulated with LPS (100 ng/ml) for indicated times in the presence or absence of gabexate mesilate (1.0 \( \times \) 10\(^{-3}\) M). Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Samples containing equal amounts of protein were resolved by 10% SDS polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated with appropriate antibodies at 4°C overnight and subsequently with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Specific proteins were visualized using enhanced chemiluminescence system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).

Electrophoretic Mobility Shift Assay (EMSA). Human monocytes (1 \( \times \) 10\(^5\) cells/assay) were stimulated with LPS (100 ng/ml) for 1 h in the presence or absence of gabexate mesilate (1.0 \( \times \) 10\(^{-3}\) M). Nuclear extracts were prepared as described previously (Cheshire and Baldwin, 1997). Double-stranded oligonucleotides containing the sequences corresponding to NF-\( \kappa \)B consensus site (5’-AGTTGAGGGAAGTGACTTCCAGGC-3’-TCAACTCTTCCCTGAAGGTTCCG-5’) and AP-1 consensus site (5’-CGGTTGATGATCGAGGGACTTTCCCAGGC-3’-GCAGGAACTACTCAGTCGGCCTT-5’) were end-labeled with digoxigenin. Binding reactions were carried out in a final volume of 15 \( \mu \)l containing 0.8 ng of digoxigenin-labeled double-stranded NF-\( \kappa \)B and AP-1 consensus oligonucleotides, 5 \( \mu \)g of nuclear extract, 1 \( \mu \)g of poly (dI-dC), and binding buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 1 mM dithiothreitol, 2% Tween 20, and 30 mM KCl]. The mixtures were incubated for 15 min at room temperature, followed by another 10 min on ice. Samples were subjected to electrophoresis in 6% non-denaturing polyacrylamide gel in a 0.5× Tris-borate-EDTA buffer system. The gel was transferred to a nylon membrane (Roche Diagnostics) by electroblotting. The membrane was then treated with anti-digoxigenin-AP for 30 min and visualized using the chemiluminescent substrate for alkaline phosphatase (CSPD; Roche Diagnostics).

Detection of Specific Binding of NF-\( \kappa \)B and AP-1 to DNA by ELISA. Analysis of the specific binding of p65/p50 and c-Fos/c-Jun to their DNA consensus oligonucleotides was performed in nuclear extracts using the ELISA-based TransAM NF-\( \kappa \)B p65/p50 and AP-1 c-Fos/c-Jun transcription factor assay kits (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. This method is based on nonisotopic quantitative ELISA-based analysis and was reported to be more sensitive than EMSA (Shen et al., 2002).

Statistical Analysis. Data are presented as mean ± S.D. values. Results were compared by analysis of variance followed by Scheffe’s post hoc test. A level of \( p < 0.05 \) was accepted as statistically significant.

Results

Effect of Gabexate Mesilate on TNF-\( \alpha \) Production by LPS-Stimulated Monocytes. Human monocytes were stimulated with LPS in the presence or absence of various concentrations of gabexate mesilate to examine the effect of gabexate mesilate on the TNF-\( \alpha \) production. The LPS-induced increase in TNF-\( \alpha \) production by monocytes was significantly inhibited by gabexate mesilate at a concentration of 1.0 \( \times \) 10\(^{-3}\) M (Fig. 1). Cell viability was not changed 4 h after incubation of monocytes with 1.0 \( \times \) 10\(^{-3}\) M of gabexate mesilate in the presence or absence of LPS (data not shown).

Effect of Gabexate Mesilate on LPS-Induced Increase of Binding of NF-\( \kappa \)B to DNA. NF-\( \kappa \)B is an important transcription factor in the induction of TNF-\( \alpha \) transcription in response to LPS (Yao et al., 1997). We examined whether gabexate mesilate inhibited LPS-induced increase of the binding of NF-\( \kappa \)B to DNA. Human monocytes were stimulated with LPS for 1 h in the presence or absence of gabexate mesilate. Nuclear extracts were assayed for NF-\( \kappa \)B activation by EMSA using an oligonucleotide that contains the consensus NF-\( \kappa \)B binding site. Analysis of the nuclear extract from LPS-stimulated monocytes demonstrated an increase in the binding of NF-\( \kappa \)B to DNA compared with the nuclear extract from unstimulated cells (Fig. 2). Pretreat-
NF-κB →

LPS - + +
Gabexate mesilate - + +

Fig. 2. Effect of gabexate mesilate on LPS-induced increase of the binding of NF-κB to DNA. Human monocytes were preincubated with or without gabexate mesilate (1.0 × 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/ml) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 μg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus NF-κB binding site. As shown in Fig. 3, A and B, the specific binding of p65 and p50 to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells. Pretreatment with gabexate mesilate significantly inhibited the LPS-induced increase of the specific binding of p65 and p50 to DNA (Fig. 3, A and B).

Effect of Gabexate Mesilate on LPS-Induced Phosphorylation and Degradation of IκBα. Activation of NF-κB was shown to require degradation of IκB, which normally binds to NF-κB in the cytoplasm to prevent nuclear translocation (Baldwin, 1996). To clarify whether gabexate mesilate inhibited the LPS-induced degradation of IκBα, we investigated the effect of gabexate mesilate on the cytoplasmic level of IκBα in monocytes stimulated with LPS by Western blot analysis. Treatment with LPS resulted in the degradation of IκBα within 15 min, followed by an increase at 30 min after stimulation (Fig. 4A). Pretreatment of cells with gabexate mesilate inhibited IκBα degradation at 15 min after the addition of LPS (Fig. 4B).

To examine whether inhibition of LPS-induced IκBα degradation by gabexate mesilate was caused by suppression of IκBα phosphorylation, we determined the cytoplasmic level of the phosphorylated form of IκBα by Western blot analysis using an antibody against the phosphorylated form of IκBα. We were able to detect the phosphorylated form of IκBα before its degradation (Fig. 5A). Stimulation with LPS induced IκBα phosphorylation within 5 min, followed by a decrease of degradation of IκBα (Fig. 5A). Pretreatment with gabexate mesilate inhibited the phosphorylation of IκBα at 5 min after LPS stimulation (Fig. 5B). These results suggested that gabexate mesilate inhibited the binding of NF-κB to DNA by preventing IκBα phosphorylation and its subsequent degradation in LPS-stimulated human monocytes.

Effect of Gabexate Mesilate on LPS-Induced Increase of Binding of AP-1 to DNA. AP-1, a transcription factor, was shown to play an important role in the production of TNF-α in LPS-stimulated monocytes by increasing its transcription (Hambleton et al., 1996). Therefore, we also examined the effect of gabexate mesilate on LPS-induced binding of AP-1 to DNA. Human monocytes were treated with LPS for 1 h in the presence or absence of gabexate mesilate. Nuclear extracts were prepared and examined for AP-1 activation by EMSA using an oligonucleotide containing the consensus AP-1 binding site. As shown in Fig. 6, AP-1 binding of human monocytes was increased at 5 min after LPS stimulation (Fig. 6A). Pretreatment with gabexate mesilate inhibited AP-1 binding of DNA (Fig. 6B).
binding to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells. Pretreatment with gabexate mesilate significantly inhibited LPS-induced binding of c-Fos and c-Jun to DNA (Fig. 7). We further analyzed the LPS-induced increase in the specific binding of c-Fos and c-Jun to the consensus AP-1 site of the target DNA. Specific binding of c-Fos and c-Jun to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells. Pretreatment with gabexate mesilate significantly inhibited the LPS-induced increase of the specific binding of c-Fos and c-Jun to DNA (Fig. 7).

**Effect of Gabexate Mesilate on LPS-Induced Phosphorylation of JNK and p38 MAPK.** Activation of JNK by LPS or proinflammatory cytokines was found to be prominently involved in the activation of AP-1 (Karin et al., 1997). To clarify whether gabexate mesilate inhibited LPS-induced phosphorylation of JNK and p38 MAPK, cytoplasmic extracts were prepared and examined by Western blot analysis using a phospho-specific IκBα antibody recognizing phosphorylation at Ser32. Three independent experiments gave similar results and typical results were shown.

**Fig. 5.** Effect of gabexate mesilate on LPS-induced phosphorylation of IκBα. A, human monocytes were stimulated with LPS (100 ng/ml) for indicated times. Cytoplasmic extracts were analyzed by Western blot analysis using a phospho-specific IκBα antibody recognizing phosphorylation at Ser32. Three independent experiments gave similar results and typical results were shown. B, human monocytes pretreated with or without gabexate mesilate (1.0 × 10^{-3} M) for 30 min were stimulated with LPS (100 ng/ml) for 5 min. Cytoplasmic extracts were analyzed by Western blot analysis using a phospho-specific IκBα antibody recognizing phosphorylation at Ser32. Three independent experiments gave similar results and typical results were shown.

**Fig. 6.** Effect of gabexate mesilate on LPS-induced increase of the binding of AP-1 to DNA. Human monocytes were preincubated with or without gabexate mesilate (1.0 × 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/ml) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 μg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus AP-1 binding site. Three independent experiments gave similar results and typical results were shown.
AP-1 activation through inhibition of JNK phosphorylation, we investigated the effect of gabexate mesilate on phosphorylation of JNK at the cytoplasmic level in cells stimulated with LPS by Western blot analysis. Because dual phosphorylation of Thr183/Tyr185 of JNK is essential for the kinase activity (Derijard et al., 1994), we used an antibody that recognizes these phosphorylated residues in the analysis. Phosphorylation of JNK was increased after LPS stimulation, reaching its maximum level at 30 min after the stimulation, and then decreasing gradually (Fig. 8A). Pretreatment with gabexate mesilate inhibited JNK phosphorylation at 30 min after LPS stimulation (Fig. 8B).

Activation of p38 MAPK has been shown to contribute to AP-1 activation (Minden and Karin, 1997). Therefore, we examined the effect of gabexate mesilate on LPS-induced phosphorylation of p38 MAPK by Western blot analysis. Because dual phosphorylation at Thr180/Tyr182 is required for p38 MAPK activation (Raingeaud et al., 1995), we used an antibody that recognizes these phosphorylated residues in this analysis. Stimulation of monocytes with LPS resulted in phosphorylation of p38 MAPK within 10 min. The cytoplasmic level of phosphorylated p38 MAPK reached a peak at 15 min after stimulation and then gradually decreased (Fig. 9A). Gabexate mesilate significantly inhibited p38 MAPK phosphorylation at 15 min after LPS stimulation (Fig. 9B). These observations indicated that gabexate mesilate inhibited the binding of AP-1 to DNA by inhibiting phosphorylation of both JNK and p38 MAPK in LPS-stimulated human monocytes.

Effect of Gabexate Mesilate on TNF-α Production and Increase in Binding of NF-κB to DNA in Monocytes Stimulated with a Low Concentration of LPS. We further examined whether various concentrations of gabexate mesilate lower than 1.0 × 10⁻³ M also inhibit LPS-induced TNF-α production in human monocytes stimulated with 1 ng/ml of LPS, a much lower concentration of LPS than that used in the present study. The LPS-induced increase in TNF-α production by monocytes was significantly inhibited by gabexate mesilate at the concentration of 1.0 × 10⁻⁶ M (Fig. 10), a concentration lower than that required to inhibit TNF-α production by monocytes stimulated with 100 ng/ml of LPS.

We also analyzed the effect of various concentrations of gabexate mesilate lower than 1.0 × 10⁻³ M on the LPS-induced increase in the binding of NF-κB to DNA in human monocytes stimulated with 1 ng/ml LPS. Nuclear extracts were analyzed for NF-κB activation by EMSA using an oligonucleotide that contains the consensus NF-κB binding site. As shown in Fig. 11, the binding of NF-κB to DNA was significantly increased in the nuclear extract from LPS-stimulated monocytes compared with that seen in the nuclear extract from unstimulated cells. Pretreatment of monocytes with various concentrations of gabexate mesilate lower than 1.0 × 10⁻³ M significantly inhibited LPS-induced increase in the binding of NF-κB to DNA (Fig. 11).

Discussion

In the present study, we demonstrated that gabexate mesilate inhibited TNF-α production by LPS-stimulated human monocytes through inhibition of the activation of both NF-κB and AP-1.

Gabexate mesilate is a synthetic serine protease inhibitor that inhibits various serine proteases generated during the coagulation cascade and the inflammatory process (Tamura...
versus LPS (H11002 phenylalanine chloromethyl ketone and proteins (Baldwin, 1996). Disruption of the NF-κB expression of macrophages induced by LPS by inhibiting degradation of IkB, thereby inhibiting TNF-α production in monocytes.

Because phosphorylated IkBα is degraded by a proteasome, a multisubunit protease complex (Finco and Baldwin, 1995), it is possible that gabexate mesilate prevented the nuclear translocation of NF-κB by inhibiting degradation of IkB through inhibition of some proteases in the proteasome. In fact, proteasome inhibitors, such as N-benzoyloxycarbonyl-Ile-Glu (O-β-Bu)-Ala-leucinal and N-acetyl-Leu-Leu-norleucinal were shown to inhibit LPS-induced degradation of IkBα and to block the production of TNF-α by human monocytes and by human monocyte leukemia cell line cells (Haas et al., 1998).

The transcription factor AP-1 can also be activated by LPS, leading to enhancement of TNF-α transcription (Hambleton et al., 1996). We showed that gabexate mesilate inhibited LPS-induced binding of AP-1 to target sites of DNA in human monocytes. AP-1 has been identified as a target of MAPK signaling pathways (Karin, 1995). The responses to LPS or proinflammatory cytokines are mostly dependent on JNK and p38 MAPK pathways, two MAPK cascades (Karin, 1995). Activation of JNK and p38 MAPK by dual phosphorylation was shown to enhance the transcriptional activity of AP-1 (Whitmarsh and Davis, 1996); whereas gabexate mesilate has been demonstrated to inhibit LPS-induced phosphorylation of JNK and p38 MAPK. The findings of this study suggest that gabexate mesilate inhibited AP-1 activation in LPS-stimulated human monocytes by preventing phosphorylation of both JNK and p38 MAPK.

Several lines of evidence from in vitro studies indicated that both toll-like receptor (TLR)2 and TLR4 expressed on the monocytic cell surface play critical roles in both activation of NF-κB and the expression of genes for various cytokines (Medzhitov et al., 1997; Yang et al., 1998). However, a previous study (Iwadou et al., 2002) demonstrated that gabexate mesilate inhibited TNF-α production in PBMCs stimulated with LPS without down-regulating TLR4 expression, suggesting that the inhibitory effect of gabexate mesilate on LPS-induced TNF-α production could be due to the inhibition of intracellular signaling pathways. They also showed that gabexate mesilate did not down-regulate the expression of TLR2 in PBMCs stimulated with staphylococcal enterotoxin B (Iwadou et al., 2002), suggesting that expression of TLRs involved in the cytokine production by monocytes could not be affected by gabexate mesilate. Activation of NF-κB and AP-1 may represent two distinct but interactive signal transduction pathways involved in LPS-induced inflammatory responses. Cross talk occurs between the upstream pathways of NF-κB and MAPK (Stein et al., 1993). Thus, it is possible that gabexate mesilate inhibits LPS-induced phosphorylation of IkBα, JNK and p38 MAPK by inhibiting the upstream pathways. This possibility should be examined in future studies.

The chemical structure of gabexate mesilate is similar to that of CNI-1493, a tetravalent guanylhydrazone, that was shown to be a competitive inhibitor of cytokine-inducible L-arginine transport and nitric oxide production in macrophages activated with LPS and interferon-γ (Bianchi et al., 1995). CNI-1493 was also shown to inhibit the production of TNF-α by human monocytes (Bianchi et al., 1996) and this

Fig. 10. Effect of gabexate mesilate on TNF-α production by monocytes stimulated with a low concentration of LPS. Human monocytes were preincubated with various concentrations of gabexate mesilate for 30 min and then stimulated with LPS (1 ng/ml) for 4 h. Supernatants were collected and TNF-α levels were determined by ELISA. **, p < 0.01 versus LPS (−); ††, p < 0.01 versus LPS (+).

Fig. 11. Effect of gabexate mesilate on increase of the binding of NF-κB to DNA in monocytes stimulated with a low concentration of LPS. Human monocytes were preincubated with various concentrations of gabexate mesilate for 30 min and then stimulated with LPS (1 ng/ml) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 μg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus NF-κB binding site. Three independent experiments gave similar results and typical results were shown.
effect could be at least partly mediated by inhibition of p38 MAPK (Tracey, 1998). Because gabexate mesilate also inhibited activation of p38 MAPK in LPS-stimulated human monocytes as shown in the present study, these compounds might inhibit the monocyte TNF-α production by the similar molecular mechanism(s).

We demonstrated in rats that gabexate mesilate reduced ischemia/reperfusion-induced liver injury and compression trauma-induced spinal cord injury in which the ischemia/reperfusion mechanism is critically involved (Taoka et al., 1997; Harada et al., 1999). Reactive oxygen species play an important role in the activation of monocytes to increase TNF-α production, thus contributing to the development of ischemia/reperfusion-induced tissue injury (Volk et al., 1999). AP-1 has been shown to be implicated in the ischemia/reperfusion-induced increase of TNF-α production (Yeh et al., 2000), and inhibition of AP-1 activation by gabexate mesilate might explain the therapeutic effect in the tissue injury induced by ischemia/reperfusion.

The concentration of gabexate mesilate required to inhibit LPS-induced TNF-α production in human monocytes was 1.0 × 10^{-8} M when human monocytes were stimulated with LPS at a concentration of 100 ng/ml. The plasma level of gabexate mesilate in humans intravenously administered a therapeutic dose of gabexate mesilate (2 mg/kg) was 2.6 × 10^{-7} M (Y. Sakai, unpublished observation), which is much lower than the concentration required to inhibit TNF-α production in vitro. Such a high concentration of gabexate mesilate (1.0 × 10^{-3} M) could not be attained in septic patients given a therapeutic dose of gabexate mesilate. In this study, human monocytes were stimulated with LPS at a concentration of 100 ng/ml, which is much higher than that seen in plasma of septic patients (Opal et al., 1999). Gabexate mesilate inhibited both TNF-α production and binding of NF-κB to DNA at a concentration of 1.0 × 10^{-6} M when monocytes were stimulated with LPS at a concentration of 1 ng/ml. Thus, it is possible that gabexate mesilate inhibits the monocyte TNF-α production in septic patients whose plasma LPS levels might be much lower than 1 ng/ml.

In this study, human monocytes were incubated with gabexate mesilate 30 min before LPS stimulation. We previously demonstrated in rats that posttreatment as well as pretreatment of animals with gabexate mesilate prevented post-traumatic spinal cord injury in which TNF-α plays a causative role, suggesting that posttreatment of gabexate mesilate could be effective in vivo (Taoka et al., 1997). These observations strongly suggest that gabexate mesilate may be potential for inhibition of TNF-α production in the clinical setting.

Gabexate mesilate has been used to treat patients with DIC associated with sepsis probably due to its anticoagulant properties (Tenaaka et al., 1983). Because TNF-α is critically involved in the activation of the extrinsic pathway of the coagulation system, thereby inducing DIC in the pathological condition of sepsis (Okajima, 2001), inhibition of TNF-α production by gabexate mesilate might at least partly contribute to reduce the coagulation abnormalities in patients with sepsis. TNF-α also plays an important role in the development of pulmonary vascular injury by activating neutrophils and endothelial cells (Zimmerman et al., 1999). Preliminary experiments showed that gabexate mesilate inhibited the activation of endothelial cells by inhibiting activation of NF-κB in cultured human umbilical vein endothelial cells. These observations indicated that inhibition of TNF-α production by gabexate mesilate might be useful in preventing the sepsis-associated organ failure such as ARDS, which adversely affects the outcome of patients with sepsis.

In conclusion, our results suggested that gabexate mesilate exerts its therapeutic effects in septic patients not only due to its anticoagulant activity, but also by inhibiting TNF-α production by monocytes. Such properties of gabexate mesilate might be useful in treating patients with sepsis for reducing organ failure as well as the coagulation abnormalities.

References


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